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Endothelial Differentiation Potential of Human Monocyte-Derived Multipotential Cells

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ABSTRACT

We previously reported a unique $CD14^+CD45^+CD34^+$ type I $collagen^+$ cell fraction derived from human circulating $CD14^+$ monocytes, named monocyte-derived multipotential cells (MOMCs). This primitive cell population contains progenitors capable of differentiating along the mesenchymal and neuronal lineages. Here, we investigated whether MOMCs can also differentiate along the endothelial lineage. MOMCs treated with angiogenic growth factors for 7 days changed morphologically and adopted a caudate appearance with rod-shaped microtubulated structures resembling Weibel-Palade bodies. Almost every cell expressed CD31, CD144, vascular endothelial growth factor (VEGF) type 1 and 2 receptors, Tie-2, von Willebrand factor (vWF), endothelial nitric-oxide synthase, and CD146, but CD14/CD45 expression was markedly downregulated. Under these culture conditions, the MOMCs continued to proliferate for up to 7 days. Functional characteristics, including vWF release upon histamine stimulation and upregulated expression of VEGF and VEGF type 1 receptor in response to hypoxia, were indistinguishable between the MOMC-derived endothelial-like cells and cultured mature endothelial cells. The MOMCs responded to angiogenic stimuli and promoted the formation of mature endothelial cell tubules in Matrigel cultures. Finally, in xenogenic transplantation studies using a severe combined immunodeficient mouse model, syngeneic colon carcinoma cells were injected subcutaneously with or without human MOMCs. Cotransplantation of the MOMCs promoted the formation of blood vessels, and more than 40% of the tumor vessel sections incorporated human endothelial cells derived from MOMCs. These findings indicate that human MOMCs can proliferate and differentiate along the endothelial lineage in a specific permissive environment and thus represent an autologous transplantable cell source for therapeutic neovasculogenesis. STEM CELLS *2006;24:2733–2743*

INTRODUCTION

Circulating cells derived from bone marrow have been reported to promote the repair of ischemic damage in organs, possibly by inducing and modulating vasculogenesis in ischemic areas or by stimulating the re-endothelialization of injured blood vessels [1, 2]. Several studies have highlighted the contribution to neovasculogenesis in adults of circulating endothelial cell progenitors, which are characterized by the expression of CD34 and vascular endothelial growth factor (VEGF) type 2 receptor (VEGFR2) [3, 4]. Recently, Harraz et al. reported that $CD14^+$ monocytes also have the potential to be incorporated into the endothelium of blood vessels in mouse ischemic limbs and to transdifferentiate into endothelial cells [5]. In addition, recent studies have shown that human $CD14^+$ monocytes coexpress endothelial lineage markers and form cord-like structures in vitro in response to a combination of angiogenic factors [6, 7]. On the other hand, several lines of evidence indicate that endothelial progenitor cells (EPCs) obtained by culturing peripheral blood mononuclear cells (PBMCs) in media favoring endothelial differentiation, which were originally reported as circulating angioblasts [3], are composed predominantly of endothelial-like cells (ELCs) derived from circulating monocytes [8, 9]. These findings indicate a potential developmental relationship between monocytes and endothelial cells and suggest that the monocyte population may be recruited for vasculogenesis and may represent an endothelial precursor population.

Recently, we identified a human cell population termed monocyte-derived multipotential cells (MOMCs; previously termed monocyte-derived mesenchymal progenitors) that has a unique phenotype that is positive for CD14, CD45, CD34, and

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type I collagen [10]. This cell population contains progenitors that can differentiate into several distinct mesenchymal cell types, including bone, cartilage, fat, and skeletal and cardiac muscle cells, as well as neurons [10–12]. MOMCs are generated in vitro by culturing circulating CD14$^+$ monocytes on fibronectin in the presence of soluble factors derived from circulating CD14$^-$ cells. MOMCs express several endothelial markers, including CD144/vascular endothelial (VE)-cadherin and VEGF type 1 receptor (VEGFR1), and have the ability to take up acetylated low-density lipoproteins (AcLDLs). In this study, the endothelial differentiation potential of human MOMCs was examined, and the capacity to induce in vitro and in vivo vascularization was compared between MOMCs and ELCs generated from circulating CD14$^+$ monocytes in the EPC induction culture.

Materials and Methods

Preparation of MOMCs

Human MOMCs were generated from the peripheral blood of healthy adult individuals, as described previously [10]. Briefly, PBMCs were resuspended in low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com), 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin, spread at a density of 2×10^6 cells per milliliter on plastic plates that had been previously treated with 10 μg/ml human fibronectin (Sigma-Aldrich), incubated overnight at 4°C, and cultured without any additional growth factors at 37°C with 5% CO_2 in a humidified atmosphere. The medium containing floating cells was exchanged with fresh medium every 3 days. After 7–10 days of culture, the adherent cells were collected as MOMCs and used in the following experiments. All blood samples were obtained after the subjects gave their written informed consent, as approved by the Institutional Review Board.

In some experiments, circulating CD14$^+$ monocytes were separated from PBMCs using an anti-CD14 monoclonal antibody (mAb) coupled to magnetic beads (CD14 MicroBeads; Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com) followed by magnetic cell sorting (MACS) column separation according to the manufacturer's protocol. A fraction enriched in CD14$^+$ cells was also prepared from cultured MOMCs using anti-CD14 mAb-coupled magnetic beads. Flow cytometric analysis revealed that these sorted fractions contained >99% CD14$^+$ cells. MOMCs were generated from the freshly isolated CD14$^+$ monocytes by culturing them alone on fibronectin-coated plates in CD14$^-$ cell-conditioned medium, which was prepared by culturing CD14$^-$ cells on fibronectin-coated plates overnight [10]. PBMCs depleted of CD34$^+$ cells were also prepared, using anti-CD34 mAb-coupled MACS beads, and used in the culture for MOMC differentiation.

Other Cell Types

Macrophages were prepared by culturing adherent PBMCs on plastic plates in Medium 199 (Sigma-Aldrich) supplemented with 20% FBS and 4 ng/ml macrophage-colony stimulating factor (R&D Systems Inc., Minneapolis, http://www.rndsystems.com) for 7 days. Human umbilical vein endothelial cells (HUVECs) and human pulmonary artery endothelial cells (HPAECs) were purchased from Cambrex (Baltimore, http://www.cambrex.com). Primary cultures of human fibroblasts were established from the skin biopsy of a healthy volunteer and maintained in low-glucose DMEM with 10% FBS.

Endothelial Induction Culture

The endothelial induction culture was carried out using the same medium as for the generation of EPCs [8, 9]. Specifically, MOMCs or freshly isolated CD14$^+$ monocytes (40%–50% confluent) were cultured on fibronectin-coated plastic plates or chamber slides for up to 14 days in endothelial cell basal medium-2 (EBM-2) (Clonetics) supplemented with EBM-2 MV SingleQuots containing 5% FBS, VEGF, basic fibroblast growth factor (bFGF), epidermal growth factor, insulin-like growth factor-1, heparin, and ascorbic acid. The medium was exchanged with fresh medium every 3–4 days.

Transmission Electron Microscopy

MOMCs grown in endothelial differentiation or control cultures were immediately fixed with 2.5% glutaraldehyde, postfixed in 2% osmium tetroxide, dehydrated in a series of graded ethanol solutions and propylene oxide, and embedded in epoxy resin. The cells were then thin-sectioned with a diamond knife. Sections in the range of gray to silver were collected on 150-mesh grids, stained with uranyl acetate and lead citrate, and examined under a JEOL-1200 EXII electron microscope (Jeol, Tokyo, http://www.jeol.com).

Flow Cytometric Analysis

Fluorescence cell staining was performed as described previously [10]. The cells were stained with a combination of the following mouse mAbs, which were either unconjugated or conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), or PC5: anti-CD14, anti-CD34, anti-CD40, anti-CD45, anti-CD80, anti-CD105, anti-CD106, anti-CD117/c-kit (Beckman Coulter, Fullerton, CA, http://www.beckmancoulter.com), anti-CD34, anti-CD133 (Miltenyi Biotec), anti-CD54, anti-CD86 (Ancell, Bayport, MN, http://www.ancell.com), anti-CD31, anti-VEGFR1, anti-VEGFR2, anti-human leukocyte antigen (HLA)-DR (Sigma-Aldrich), anti-CD144, anti-CD146/H1P12, or anti-type I collagen (Chemicon, Temecula, CA, http://www.chemicon.com). When unconjugated mAbs were used, goat anti-mouse IgG $F(ab')_2$ conjugated to FITC or PE (Beckman Coulter) was used as a secondary antibody. For intracellular type I collagen staining, the cells were permeabilized and fixed using the IntraPrep permeabilization reagent (Beckman Coulter). Negative controls were cells incubated with an isotype-matched mouse mAb to an irrelevant antigen. The cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Diego, http://www.bdbiosciences.com) using CellQuest software.

Immunohistochemistry on Cultured Cells

The diaminobenzidine (DAB) staining of cultured cells was performed as described [10]. The primary antibodies used were rabbit polyclonal anti-Tie-2 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com) or one of the following mouse mAbs: anti-CD45, anti-vimentin (Dako, Carpinteria, CA, http://www.dako.com), anti-CD34 (Ancell),

anti-CD105 (Beckman Coulter), anti-type I collagen, anti-CD144, anti-CD146, anti-human nuclei (Chemicon), anti-VEGFR1, anti-VEGFR2 (Sigma-Aldrich), anti-von Willebrand factor (anti-vWF), and anti-endothelial nitric-oxide synthase (anti-eNOS) (BD Biosciences). Negative controls were cells incubated with normal rabbit IgG or isotype-matched mouse mAb to an irrelevant antigen, instead of the primary antibody. Biotin-labeled anti-mouse or rabbit IgG antibodies combined with a streptavidin-horseradish peroxidase complex (Nichirei, Tokyo, http://www.nichirei.co.jp/english) were used for DAB staining. Nuclei were counterstained with hematoxylin. To enumerate the proportion of cells staining positive for a given marker, at least 300 cells per culture were evaluated.

Uptake of AcLDL

Cultured adherent cells were labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled AcLDL (Dil-AcLDL) (2.5 μg/ml) (Molecular Probes Inc., Eugene, OR, http://probes.invitrogen.com) for 1 hour at 37°C, and AcLDL uptake was evaluated by flow cytometry and by fluorescence microscopy (IX71; Olympus, Tokyo, http://www.olympus-global.com).

Analysis of mRNA Expression

The expression of mRNA was examined using reverse transcription (RT) combined with polymerase chain reaction (PCR) as described [10]. Total RNA was extracted from HUVECs, monocyte-derived ELCs, and mouse colon carcinoma cell line CT-26, and human MOMCs that had or had not been induced to differentiate for 3, 5, 7, or 14 days, using the RNeasy kit (Qiagen, Valencia, CA). First-strand cDNA synthesized from the total RNA was subjected to PCR amplification using a panel of specific primers (supplemental online Table 1) [6, 10]. The PCR products were resolved by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining.

Cell Proliferation Study

Proliferating MOMCs were detected by bromodeoxyuridine (BrdU) incorporation as described previously [12]. Briefly, MOMCs were cultured in the presence of 10 μM BrdU (Sigma-Aldrich) for 2 hours before staining. After cell fixation and DNA denaturation, the cells were incubated with a rat anti-BrdU mAb (Abcam, Cambridge, U.K., http://www.abcam.com) and a mouse mAb to human nuclei or eNOS followed by incubation with AlexaFluor 488 mouse-specific IgG and AlexaFluor 568 rat-specific IgG (Molecular Probes). Cells were observed under a confocal laser fluorescence microscope (LSM5 PASCAL; Carl Zeiss, Göttingen, Germany, http://www.zeiss.com). To enumerate the proliferating human MOMCs, the number of BrdU-positive nuclei in the total number of nuclei was calculated. Apoptotic cells were also detected by incubating unfixed cells with propidium iodide (Sigma-Aldrich).

Histamine-Mediated Release of vWF

MOMCs after endothelial differentiation treatment and HUVECs were incubated with 10 μM histamine (Sigma-Aldrich) in FBS-free low-glucose DMEM for 25 minutes. Untreated and treated cells were fixed with 10% formalin and stained with a mouse anti-vWF mAb (BD Biosciences) followed by incubation with AlexaFluor 568 mouse-specific IgG (Molecular Probes) and then with FITC-conjugated mouse anti-human nuclear mAb (Chemicon).

Changes in Gene Expression Profiles in Response to Hypoxia

MOMCs after endothelial differentiation treatment and HPAECs were incubated at 37°C in 21% or 1% oxygen for 24 hours [13]. The cells were then harvested and subjected to mRNA expression analyses using RT-PCR and the TaqMan quantitative PCR system (Applied BioSystems, Foster City, CA, http://www.appliedbiosystems.com). A combination of primers and a probe specific for VEGFR1 were designed as follows: forward primer, 5′-AACACAAGATGGCAAATCAGGAT-3′; reverse primer, 5′-GGCGCCACCGCTTAAGA-3′; and probe, 5′-(FAM)-AGGTGAAAAGATCAAGAAACGTGTGAAAAC-TCC-(TAMRA)-3′, whereas those for VEGF, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β-actin were purchased from Applied BioSystems. Expression levels were calculated from a standard curve generated by plotting the amount of PCR product against the serial amount of input normoxic HPAEC cDNA and were expressed relative to the level of the same gene under normally oxygenated conditions.

In Vitro Vascular Tube Formation

The formation of endothelial tubular structures was studied in vitro in Matrigel cultures. Briefly, MOMCs, MOMC-derived ELCs, monocyte-derived ELCs, or cultured dermal fibroblasts (10^4 or 10^5) in EBM-2 were seeded onto 24-well plates coated with Matrigel (BD Biosciences) with or without a suboptimal number of HUVECs (10^3), which was insufficient to form typical tube structures. HUVECs (10^4) cultured with HUVECs (10^3) were used as a positive control. The cells were cultured at 37°C for 24 hours and observed with an IX71 inverted microscope. The total tube length was calculated from 10 randomly selected low-power fields for each experiment. In some experiments, MOMCs (10^4) were labeled with the green fluorescent cell linker PKH67 (Sigma-Aldrich) or Dil-AcLDL before being added to the Matrigel culture with unlabeled HUVECs (10^3). Dil-AcLDL-labeled MOMCs cultured in Matrigel for 1 or 3 days were collected using a Cell Recovery Solution (BD Biosciences), cytospun, and stained with mouse anti-eNOS or anti-CD45 mAb, followed by incubation with AlexaFluor 488 mouse-specific IgG and DAPI.

Mouse Model for In Vivo Tumor Neovascularization

All procedures were performed on severe combined immunodeficient (SCID) mice obtained from Charles River Japan (Yokohama, Japan, http://www.crj.co.jp), which were kept in specific pathogen-free conditions according to the Keio University Animal Care and Use Committee guidelines. Syngeneic murine colon carcinoma CT-26 cells (2.5×10^5) were transplanted subcutaneously into the back of SCID mice, with or without MOMCs, MOMC-derived ELCs (10^4 or 10^5), monocyte-derived ELCs, monocytes, or macrophages (10^5). Subcutaneous tumor sizes were measured by external caliper, and tumor volume was calculated with the following formula: volume $= 0.5 \times$ longest diameter $\times$ (shortest diameter)^2. Subcutaneous tumors were removed 10 days after the transplantation, and then formalin-fixed, paraffin-embedded specimens were

sectioned and stained with hematoxylin and eosin. The number of erythrocyte-bearing blood vessels was counted in 10 independent fields, and the results were expressed as the number per 1 mm^3. Frozen sections (10-μm thick) of the tumor were subjected to immunohistochemistry, in which the slides were incubated with a rat mAb to mouse-specific CD31 (BD Biosciences) or a rabbit polyclonal antibody to human-specific CD31 (Santa Cruz Biotechnology) in combination with a mouse mAb to human-specific CD31, HLA class I (Sigma-Aldrich), or vWF (BD Biosciences), followed by incubation with AlexaFluor 488 mouse-specific IgG and AlexaFluor 568 rat- or rabbit-specific IgG (Molecular Probes). Nuclei were counterstained with TO-PRO3 (Molecular Probes). These slides were examined with a confocal laser fluorescence microscope. The proportion of blood vessels containing human CD31-expressing endothelial cells in at least 100 blood vessel sections was calculated. Moreover, we calculated the proportion of cells expressing human CD31 in at least 100 HLA class I-positive cells.

Statistical Analysis

All continuous variables were expressed as the mean ± SD. Comparisons between two groups were tested for statistical significance using the Mann-Whitney test.

Results

Endothelial Differentiation of MOMCs

Human MOMCs took on a spindle shape in culture (Fig. 1A) and consisted of a single phenotypic population positive for CD14, CD45, CD34, and type I collagen by flow cytometric analysis (>96% homogeneous), as reported previously [10]. To investigate whether MOMCs could differentiate along the endothelial lineage, the MOMCs were replated on new fibronectin-coated plates and subjected to endothelial induction culture with EBM-2. During 7 days of culture, the morphology of the MOMCs changed from spindle-shaped to caudate or round with eccentric nuclei and extended cytoplasm (Fig. 1B). The proportion of spindle-shaped cells decreased with time, and nearly all the adherent cells had the caudate morphology on day 7. Electron microscopic analysis of MOMCs cultured under the endothelial induction conditions for 7 days revealed many cytoplasmic granules containing an electron-dense material. These rod-shaped microtubulated structures resembled Weibel-Palade bodies [14] and were detected in all the cells subjected to the endothelial induction treatment (Fig. 1C).

MOMCs cultured in EBM-2 for 7 days were then examined by immunohistochemistry for the expression of endothelial markers. As shown in Figure 1D, MOMC-derived ELCs expressed CD34, CD144, CD105, VEGFR1, VEGFR2, vWF, eNOS, CD146, and Tie-2, typical of endothelial cells. This set of endothelial markers was detected in nearly all the adherent cells, but the intensity of staining for vWF, eNOS, and CD146 was variable. The mRNA expression over time of selected endothelial markers and hematopoietic/monocytic markers in MOMCs undergoing endothelial induction treatment was further examined by RT-PCR (Fig. 1E). The mRNA expression of VEGFR1, VEGFR2, CD144, Tie-2, and vWF was upregulated during the first 7 days of culture and then plateaued, but the expression of VEGFR2 was downregulated on day 14. The expression of CD45 and CD14 was markedly downregulated

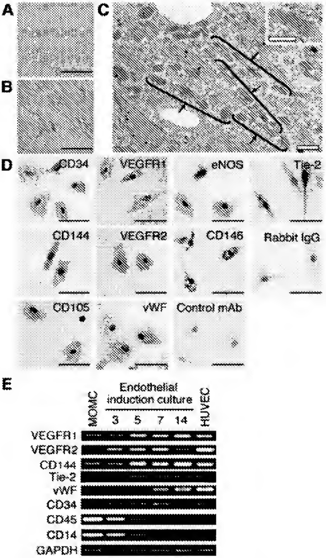

Figure 1. Morphology and protein and mRNA expression profiles of MOMC-derived endothelial-like cells. **(A, B):** Phase-contrast images of MOMCs before **(A)** and after **(B)** endothelial induction for 7 days. Scale bars = 100 μm. **(C):** A transmission electron microscopic image of MOMC-derived endothelial-like cells. Scale bar = 1 μm. Many cytoplasmic granules containing electron-dense material were observed (arrows). Inset shows an electron-dense rod-shaped inclusion at higher magnification; scale bar = 0.5 μm. Results shown are representative of 50 cells prepared in three independent experiments. **(D):** Immunohistochemical analysis of MOMCs undergoing endothelial induction for 7 days. Cells were stained with a mouse mAb or polyclonal antibody to the endothelial marker, as indicated. Controls were incubated with an isotype-matched mouse mAb to an irrelevant antigen (control mAb) or normal rabbit IgG (rabbit IgG). Nuclei were counterstained with hematoxylin. Scale bars = 50 μm. Results shown are representative of at least five independent experiments. **(E):** Reverse transcription-polymerase chain reaction analysis for mRNA expression of VEGFR1, VEGFR2, CD144, Tie-2, vWF, CD34, CD45, CD14, and GAPDH in untreated MOMCs; MOMCs with endothelial induction for 3, 5, 7, and 14 days; and HUVECs. Abbreviations: eNOS, endothelial nitric-oxide synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HUVEC, human umbilical vein endothelial cell; mAb, monoclonal antibody; MOMC, monocyte-derived multipotential cell; VEGFR, vascular endothelial growth factor receptor; vWF, von Willebrand factor.

Table 1. Protein expression profiles of MOMC-derived ELCs, various monocyte-derived cells, and HUVECs

	Circulating monocytes	MOMCs	MOMC-derived ELCs	Monocyte-derived ELCs	Macrophages	HUVECs
CD45[a,b]	++	++	+	++	++	−
CD14[a]	++	++	±	+	++	−
HLA-DR[a]	++	++	+	+	++	−
CD40[a]	+	+	+	+	++	+
CD80[a]	−	−	−	−	++	−
CD86[a]	+	+	+	+	++	−
CD54[a]	+	+	+	+	+	+
CD106[a]	−	−	+	±	−	−
CD34[a,b]	−	+	+	+	−	++
CD105/endoglin[a,b]	−	+	+	+	−	++
CD117/c-kit[a]	−	−	−	−	−	−
CD133[a]	−	−	−	−	−	−
CD31[a]	+	+	+	+	+	++
CD144/VE-cadherin[a,b]	−	+	+	+	−	+
CD146[a,b]	−	−	+	−	−	++
Flt-1/VEGFR1[a,b]	−	+	+	+	−	+
Flk-1/VEGFR2[a,b]	−	−	+	−	−	+
vWF[b]	−	−	+	±	−	++
eNOS[b]	−	−	+	+	−	++
Tie-2[b]	−	+	++	+	−	+
Type I collagen[b]	−	+	+	−	−	−
AcLDL[a,b]	+	++	++	++	++	++

Consistent results were obtained in at least five independent experiments. −, no staining; ±, weak staining, +, moderate staining; ++, strong staining.
[a]Assessed by flow cytometry.
[b]Assessed by immunohistochemistry.
Abbreviations: AcLDL, acetylated low-density lipoprotein; ELC, endothelial-like cell; eNOS, endothelial nitric-oxide synthase; HLA-DR, human leukocyte antigen-DR; HUVEC, human umbilical vein endothelial cell; MOMC, monocyte-derived multipotential cell; VE, vascular endothelial; VEGFR, vascular endothelial growth factor receptor; vWF, von Willebrand factor.

during the differentiation process, whereas CD34 expression remained constant up to day 14. Notably, the mRNA expression profile of MOMCs subjected to the endothelial induction culture for 7 days was indistinguishable from the profile of HUVECs.

These results together indicate that MOMCs can differentiate into ELCs that have morphologic and phenotypic characteristics similar to those of mature endothelial cells. This endothelial differentiation was consistently observed for MOMCs derived from 20 different healthy adult donors. In addition, a similar yield of ELCs was obtained when the same culture conditions were used for the $CD14^+$ cell-enriched MOMC fraction (>99% homogeneous), MOMCs generated from freshly isolated $CD14^+$ monocytes in $CD14^-$ cell-conditioned medium, or MOMCs generated from $CD34^+$ cell-depleted PBMCs.

Phenotypes of ELCs Derived from MOMCs and Freshly Isolated Monocytes

Several reports show that ELCs can also be generated from freshly isolated circulating $CD14^+$ monocytes by culturing them with a combination of angiogenic growth factors [5–9]. The protein expression profiles of MOMC-derived ELCs on day 7 were examined by flow cytometry and/or immunohistochemistry and compared with those of ELCs prepared by culturing freshly isolated circulating monocytes in EBM-2 for 7 days (Table 1). Representative flow cytometric analyses of the cell-surface expression of CD45, CD14, CD34, CD144, and CD146 are shown in Figure 2. Monocyte-derived ELCs displayed weak CD34 and CD144 expression and downregulated CD45 expression, as described previously [6, 7]. Comparison of the expression profiles obtained from ELCs derived from different sources showed that the MOMC-derived ELCs had a higher expression of CD34, CD144, and CD146 and a lower expression of CD45 and CD14 than the monocyte-derived ELCs. Moreover, no protein expression of VEGFR2 and vWF was apparent in the monocyte-derived ELCs under our culture and immunohistochemical conditions (Table 1).

Proliferative Capacity of MOMCs During Endothelial Differentiation

To evaluate whether MOMCs proliferate during endothelial differentiation, the number of adherent cells in the MOMC cultures with and without the endothelial induction treatment were evaluated over time (Fig. 3A). The number of MOMCs increased during culture. However, MOMC expansion in endothelial induction medium (EBM-2) was sustained up to day 7, whereas the cell expansion slowed after day 3 in cultures with regular medium (low-glucose DMEM plus 10% FBS), resulting in a statistical difference in the cell number after day 5. To confirm the difference in cell division, the proportion of dividing cells in MOMC cultures over time was evaluated by BrdU incorporation. Representative immunofluorescence images of MOMCs cultured in EBM-2 and DMEM on days 1 and 5 are shown in Figure 3B. More than 25% of the MOMCs undergoing the endothelial induction treatment incorporated BrdU on days 1 and 5, but only a small propor-

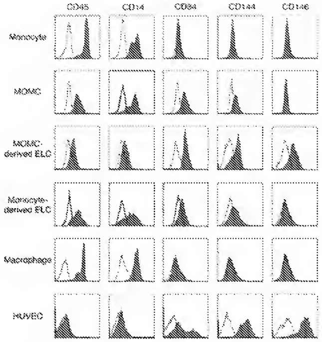

Figure 2. Flow cytometric analysis of freshly isolated circulating monocytes, undifferentiated MOMCs, MOMC-derived ELCs, monocyte-derived ELCs, macrophages, and HUVECs. MOMCs and monocytes after endothelial induction for 7 days were used as MOMC- and monocyte-derived ELCs, respectively. Cells were stained with monoclonal antibodies (mAbs) as indicated and analyzed by flow cytometry. Expression of the molecules of interest is shown as shaded histograms. Open histograms represent staining with isotype-matched control mAb. Results shown are representative of at least three independent experiments. Abbreviations: ELC, endothelial-like cell; HUVEC, human umbilical vein endothelial cell; MOMC, monocyte-derived multipotential cell.

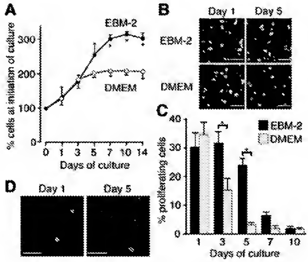

Figure 3. Proliferative capacity of MOMCs during endothelial differentiation. **(A):** The number of adherent cells in cultures of MOMCs with endothelial induction treatment (EBM-2) or without the treatment (low-glucose DMEM plus 10% fetal bovine serum [FBS]) for up to 14 days. The number of attaching cells per 1 mm^3 was counted in 10 randomly selected fields and expressed relative to the number of cells before endothelial induction. Results shown are the mean and SD from five independent donors. Asterisk indicates a statistically significant difference between the two cultures. **(B):** MOMCs were cultured for 1 or 5 days in EBM-2 or low-glucose DMEM plus 10% FBS, and bromodeoxyuridine (BrdU) incorporation during a 2-hour incubation was examined by immunohistochemistry with monoclonal antibodies (mAbs) to human nuclei (green) and BrdU (red). Yellow indicates a proliferating cell positive for both human nuclei and BrdU. Scale bars = 50 μm. **(C):** Proportion of proliferating MOMCs in culture with EBM-2 or low-glucose DMEM plus 10% FBS over time. The number of BrdU-positive nuclei divided by the total number of nuclei was calculated as the proportion of proliferating MOMCs. At least 200 cells were counted for each BrdU staining. Results are expressed as the mean and SD of four independent experiments. Asterisk indicates a statistically significant difference between the two cultures. **(D):** MOMCs were cultured for 1 or 5 days in EBM-2 and subjected to immunohistochemistry with mAbs to endothelial nitric-oxide synthase (green) and BrdU (red). Scale bars = 50 μm. Abbreviations: DMEM, Dulbecco's modified Eagle's medium; EBM-2, endothelial cell basal medium-2.

tion of the MOMCs cultured in regular medium were proliferating on day 5. Semiquantitative assessment of the $BrdU^+$ proliferating cells showed that the MOMC proliferation was greater in the endothelial induction culture than in the regular culture on days 3 and 5 (Fig. 3C). The proportion of apoptotic adherent cells positive for propidium iodide staining was <3% at all time points. When MOMCs cultured in EBM-2 were examined for BrdU incorporation and eNOS expression, nearly all cells expressing eNOS failed to incorporate BrdU at day 5 (Fig. 3D), indicating that proliferating cells are predominantly undifferentiated MOMCs.

Functional Characteristics of MOMC-Derived ELCs

We next performed a series of analyses to test whether the MOMC-derived ELCs had the functional properties of endothelial cells. First, we evaluated the capacity in vitro of MOMC-derived ELCs to release vWF in response to stimulation with histamine, which is one of the unique features of endothelial cells [15]. HUVECs and MOMC-derived ELCs were incubated with or without histamine and stained with anti-vWF and anti-nuclear mAbs (Fig. 4A). Almost half of the untreated HUVECs showed vWF throughout the cytoplasm, which disappeared after histamine treatment. Similarly, the histamine treatment resulted in a loss of vWF staining in the MOMC-derived ELCs. Another characteristic of endothelial cells is that they take up AcLDL [16]. MOMC-derived ELCs rapidly incorporated Dil-AcLDL similarly to HUVECs; however, undifferentiated MOMCs and even freshly isolated monocytes were also able to take up Dil-AcLDL (Table 1).

Endothelial cells are known to respond to hypoxia by upregulating several molecules associated with angiogenesis and glucose regulation, such as VEGF, VEGFR1 [13], and GAPDH [17]. HPAECs and MOMC-derived ELCs were exposed to a hypoxic or normoxic condition for 24 hours, and the mRNA expression levels of VEGF, VEGFR1, GAPDH, and β-actin were compared between these two cultures. The results obtained from HPAECs and MOMC-derived ELCs were concordant and showed an increased expression of VEGF, VEGFR1, and GAPDH upon exposure to the hypoxic condition (Fig. 4B).

In Vitro Angiogenic Properties of MOMCs

We next tested whether undifferentiated MOMCs or MOMC-derived ELCs could form tubular structures when plated on Matrigel. We also tested monocyte-derived ELCs, freshly iso-

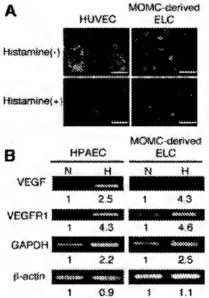

Figure 4. Functional characterization of MOMC-derived ELCs. **(A):** Histamine-mediated release of von Willebrand factor (vWF) from HUVECs and MOMC-derived ELCs. Cells were treated with or without histamine for 25 minutes and subjected to immunohistochemistry with monoclonal antibodies to vWF (red) and human nuclei (green). Representative examples of five experiments from three donors are shown. Scale bars = 50 μm. **(B):** Upregulation of mRNA for VEGF and VEGFR1 in MOMC-derived ELCs by hypoxic exposure. Cultured HPAECs and MOMC-derived ELCs were incubated in 20% O_2 (N) and 1% O_2 (H) for 24 hours, and the VEGF, VEGFR1, GAPDH, and β-actin mRNA expression was detected by reverse transcription-polymerase chain reaction (PCR). Expression levels were determined by TaqMan quantitative PCR and divided by the level of each gene under normally oxygenated conditions. Results shown are representative of three independent experiments, and the relative expression was the mean of three experiments. Abbreviations: ELC, endothelial-like cell; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H, hypoxia; HPAEC, human pulmonary artery endothelial cell; HUVEC, human umbilical vein endothelial cell; MOMC, monocyte-derived multipotential cell; N, normoxia; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

lated monocytes, and cultured dermal fibroblasts. None of the monocyte-originating cells formed typical tubular structures by themselves. Therefore, a suboptimal number of HUVECs (10^3), which induce the formation of a small number of short tubular structures when cultured alone, were cocultured with the series of monocyte-derived cells and fibroblasts (10^4) (Fig. 5A). Undifferentiated MOMCs dramatically promoted the formation of tubules in the Matrigel culture with HUVECs, but only some tubules were extended in cultures of ELCs derived from MOMCs and monocytes. Freshly isolated monocytes or fibroblasts failed to enhance the formation of tubules. Compared with the culture of HUVECs (10^3) alone, semiquantitative analysis of the tube length revealed a statistically significant enhancement in the culture of undifferentiated MOMCs with HUVECs and in the positive control culture of HUVECs (10^4) (Fig. 5B). To test whether MOMCs were integrated into the tubular structures, the cells were labeled with PKH67 before the Matrigel culture with unlabeled HUVECs. PKH67-labeled MOMCs were clearly incorporated into the tubular structure (Fig. 5C). When DiI-AcLDL-labeled MOMCs were cultured with HUVECs in Matrigel, endothelial differentiation of MOMCs was accelerated based on upregulated eNOS expression and downregulated CD45 expression at day 3 (Fig. 5D).

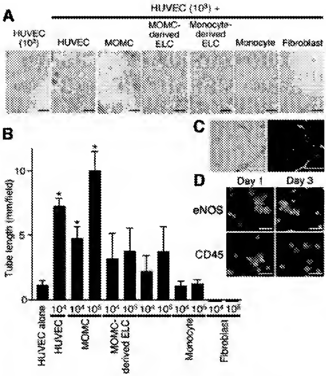

Figure 5. In vitro tubule formation promoted by various monocyte-originated cells in Matrigel culture. **(A):** HUVECs (10^3) were cultured alone or in combination with HUVECs, MOMCs, MOMC-derived ELCs, monocyte-derived ELCs, freshly isolated circulating monocytes, or cultured dermal fibroblasts (10^4) on Matrigel for 24 hours. Representative pictures of five independent experiments are shown. Scale bars = 500 μm. **(B):** Total tube length in the Matrigel cultures of HUVECs (10^3) alone and HUVECs (10^3) plus HUVECs (10^4), MOMCs, MOMC-derived ELCs, monocyte-derived ELCs, freshly isolated circulating monocytes, or cultured dermal fibroblasts (10^4 or 10^5). The combined length of the tubes was calculated from 10 randomly selected low-power fields in individual experiments, and results are expressed as the mean and SD from five independent experiments. Asterisk indicates a significantly different from HUVECs (10^3) alone. **(C):** MOMCs were previously labeled with PKH2 (10^4) and cultured on Matrigel with unlabeled HUVECs (10^3) for 24 hours. Light microscopic (top) and fluorescent (bottom) images of the same sample are shown. Scale bars = 500 μm. Results shown are representative of four independent experiments. **(D):** MOMCs were previously labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (10^4) and cultured on Matrigel with unlabeled HUVECs (10^3) for 1 or 3 days. The cells were recovered, cytospun, and examined by immunohistochemistry with monoclonal antibodies to eNOS or CD45 (green). Scale bars = 50 μm. Results shown are representative of three independent experiments. Abbreviations: ELC, endothelial-like cell; eNOS, endothelial nitric oxide synthase; HUVEC, human umbilical vein endothelial cell; MOMC, monocyte-derived multipotential cell.

In Vivo Vasculogenic Properties of MOMCs

To further examine the in vivo vasculogenic properties of various monocyte-derived cells, murine colon carcinoma CT-26 cells were transplanted into the back of SCID mice, alone or with human MOMCs, MOMC-derived ELCs, monocyte-de-

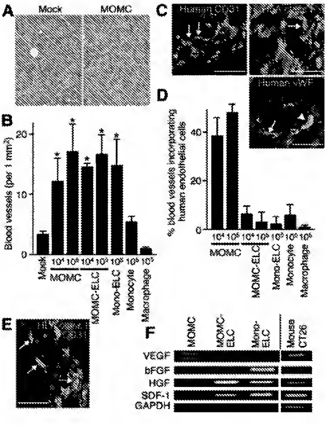

Figure 6. In vivo tumor vasculogenesis promoted by various human monocyte-derived cells in severe combined immunodeficient (SCID) mice. Murine colon carcinoma cells (CT26) were transplanted into the back of SCID mice, alone or with human MOMCs, MOMC-derived ELCs, monocyte-derived ELCs, monocytes, or macrophages, and tumor tissue sections were obtained 10 days later. **(A):** Representative tumor sections stained with hematoxylin and eosin obtained from mice receiving transplants of CT26 alone (mock) or CT26 and human MOMCs. Circles indicate blood vessels carrying erythrocytes. Scale bars = 100 μm. **(B):** Blood vessel density in tumors from mice receiving transplants of CT26 alone (mock), CT26 in combination of MOMCs, MOMC-derived ELCs (10^4 and 10^5), monocyte-derived ELCs, monocytes, and macrophages (10^5). The number of blood vessels per 1 mm^3 was calculated from 10 randomly selected fields per individual experiment, and results are expressed as the mean and SD of five independent experiments. Asterisk indicates a significant difference from mock. **(C):** Representative tumor sections from mice receiving transplants of CT26 and MOMCs, which were stained for mouse CD31 (red) and human CD31, HLA class I, or human vWF (green). Nuclei were counterstained with TO-PRO3. Arrow denotes human MOMCs that are incorporated into vascular structure and differentiated into endothelial cells, whereas arrowhead denotes human MOMCs expressing endothelial markers existing outside of the vascular lumen. Scale bars = 50 μm (human CD31) and 25 μm (HLA class I and human vWF). The results shown are representative of five experiments. **(D):** The proportion of blood vessel sections incorporating human endothelial cells in tumors from mice receiving transplants of CT26 with MOMCs, MOMC-derived ELCs (10^4 and 10^5), monocyte-derived ELCs, monocytes, and macrophages (10^5). At least 100 blood vessel sections were observed, and the proportion of vessels containing human CD31-positive endothelial cells was calculated. Results are expressed as the mean and SD of five independent experiments. **(E):** Representative tumor sections from mice receiving transplants of CT26 and MOMCs, which were stained for human CD31 (red) and HLA class I (green). Nuclei were counterstained with TO-PRO3. Yellow indicates a human cell positive for CD31. Scale bar = 50 μm. **(F):** Reverse transcription-polymerase chain reaction analysis for mRNA expression of human or mouse VEGF, bFGF, HGF, SDF-1, and GAPDH in human MOMCs, human MOMC-derived ELCs, human monocyte-derived ELCs, and murine colon carcinoma cell line CT-26. Abbreviations: bFGF, basic fibroblast growth factor; ELC, endothelial-like cell; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HGF, hepatocyte growth factor; HLA, human leukocyte antigen; MOMC, monocyte-derived multipotential cell; SDF-1, stromal cell-derived factor 1; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor.

rived ELCs, freshly isolated circulating monocytes, or macrophages. At day 10, tumor sizes in MOMC-transplanted mice tended to be larger than those in mice transplanted with macrophages (48.6 ± 7.4 vs. 39.7 ± 7.2), but this difference did not reach statistical significance. Hematoxylin-eosin-stained tumor sections obtained 10 days after transplantation from the MOMC-transplanted mice showed many blood vessels carrying erythrocytes. In contrast, only a few vessels were seen in the tumor sections from the mock-treated mice receiving CT-26 alone (Fig. 6A). A semiquantitative assessment of the number of tumor blood vessels revealed that the tumors in mice receiving CT-26 transplanted with MOMCs, MOMC-derived ELCs, and monocyte-derived ELCs had significantly more vessels than did tumors from mice receiving CT-26 alone, whereas monocytes or macrophages failed to promote tumor vasculogenesis (Fig. 6B).

All the tumors were then stained with human-specific CD31, HLA class I, or vWF mAb, combined with an anti-mouse CD31 mAb. Tumors obtained from the mice that received transplants of undifferentiated MOMCs had blood vessels that included cells expressing human-specific CD31, HLA class I, or vWF but did not coexpress mouse CD31 (Fig. 6C). These findings indicate that human MOMC-derived endothelial cells contributed to tumor vasculogenesis in vivo by being incorporated and differentiating into the endothelium, although human cells expressing endothelial markers were occasionally detected outside of the vascular lumen (Fig. 6C, arrowhead). To better address the degree of tumor vessel integration, the proportion of vessel sections containing human $CD31^+$ cells was evaluated semiquantitatively (Fig. 6D). In tumors from mice receiving human MOMC transplants, approximately 40% of the tumor vessels incorporated human endothelial cells. In contrast, the proportion of human endothelial cells was less than 10% in the tumors from mice receiving MOMC-derived or monocyte-derived ELCs, even though these cells significantly promoted blood vessel formation. However, efficiency of endothelial differentiation in transplanted MOMCs (proportion of human $CD31^+$ cells in HLA class I-positive cells) was only 9.4% ± 5.1% (n = 8; Fig. 6E).

To evaluate the source of angiogenic factors in our tumor vasculogenesis model, mRNA expression of angiogenic factors was examined in human MOMCs, MOMC-derived ELCs, monocyte-derived ELCs, and CT-26 by RT-PCR (Fig. 6F). All of these cells expressed VEGF, bFGF, hepatocyte growth factor (HGF), and stromal cell-derived factor 1 (SDF-1), and expression of bFGF, HGF, and SDF-1 in MOMCs was upregulated after endothelial induction.

Discussion

In this study, we demonstrated that MOMCs can differentiate into endothelium of a mature phenotype with typical morpho-

logic, phenotypic, and functional characteristics. This proliferation and specific differentiation was induced in MOMCs by a combination of angiogenic growth factors. MOMCs expressed CD34 and several endothelial markers, such as CD144 and VEGFR1, even untreated, but the endothelial induction treatment resulted in their morphological change to a typical caudate appearance with structures resembling Weibel-Palade bodies, the upregulation of mature endothelial markers, and the downregulation of hematopoietic/ monocytic markers. In addition, the MOMC-derived ELCs possessed in vitro functional characteristics of endothelial cells, including the release of vWF in response to the vasoactive agent histamine, the incorporation of AcLDL, and the upregulated gene expression of VEGF, VEGFR1, and GAPDH in response to hypoxia. These features were indistinguishable from those of cultured mature endothelial cells. Finally, MOMCs responded to angiogenic stimuli and promoted in vitro tubule formation in Matrigel culture and in vivo neovascularization in the setting of tumorigenesis. The MOMC's contribution of endothelial cells to vessels in the in vivo tumor model was nearly 40%, a level similar to those of other sources of endothelial progenitors [18–20], but only 10% of transplanted MOMCs differentiated into endothelial cells in vivo. It has been shown that circulating monocytes play a crucial role in neovascularization, especially in collateral vessel growth (arteriogenesis) [21, 22], and an infusion of bone marrow-derived $CD34^-CD14^+$ monocytic cells contributes to the regeneration of functional endothelium through rapid endothelialization [23]. These reports and the present study together support the idea that $CD14^+$ monocytes are not solely phagocyte precursors but also precursors for endothelium, although this fate may not be expressed during normal development in the absence of cues.

Undifferentiated MOMCs were integrated into blood vessels and differentiated into endothelium in vitro and in vivo more efficiently than did MOMC-derived ELCs and monocyte-derived ELCs, although these cell types had a similar ability to induce in vivo tumor neovascularization. The lack of integration of monocyte-derived ELCs generated in the EPC culture into a growing network of vascular endothelium is consistent with a previous study [24]. In this regard, the efficiency of neovascularization is not solely attributable to the incorporation of progenitors into newly formed vessels but is also influenced by the release of proangiogenic factors. Indeed, MOMCs, MOMC-derived ELCs, and monocyte-derived ELCs produced multiple angiogenic growth factors, and these growth factors potentially play major roles in mobilizing putative endothelial progenitors from the bone marrow and stimulating the proliferation and differentiation of residential mature endothelial cells [25]. Several cultured mature endothelial cell lines do not integrate into newly formed vessels [26, 27], and this is probably because expression levels of cell adhesion molecules and soluble factors that regulate tubular formation capacity are heterogeneous among endothelial cells [28]. Similarly, ELCs subjected to the endothelial differentiation treatment promote new blood vessel formation mainly through the secretion of proangiogenic factors. This feature is consistent with a recent study showing that bone marrow-derived hematopoietic cells are recruited to an angiogenic region in response to VEGF and contribute to vasculogenesis not being integrated as endothelial cells but existing outside of vascular lumen [29]. In contrast, undifferentiated MOMCs, which share several phenotypic features with endothelial progenitors, may contribute to neovascularization by being incorporated and differentiating into the endothelium in addition to secretion of proangiogenic factors.

During embryogenesis, the commitment of the hemangioblast, a bipotent stem cell for hematopoietic and endothelial cells, to the endothelial lineage is characterized by the sequential expression of CD144, CD31, and CD34 [30, 31]. It is reported that postnatal endothelial progenitor cells can be selected from the bone marrow and peripheral blood based on their expression of CD34, CD133, and VEGFR2 [4, 32], and these progenitors also express CD144, CD31, and Tie-2 [33]. The differentiation of these progenitor cells into mature endothelial cells is accompanied by the upregulated expression of vWF and CD146. The differentiation of circulating monocytes into the endothelial lineage via MOMCs follows the same sequence of events. Specifically, monocytes acquire the expression of CD34, CD144, and Tie-2 during their differentiation into MOMCs and are further induced to express VEGFR2 and subsequently vWF and CD146 by the endothelial induction treatment. This observation suggests that the differentiation process leading to adoption of the endothelial lineage is partly shared by monocytes and hemangioblasts, although we did not detect CD133 expression in monocytes during this differentiation process.

It is unlikely that the endothelial differentiation we observed arose from nonhematopoietic circulating precursors for endothelial cells contaminating the MOMC population. In this regard, peripheral blood contains $CD34^+CD133^+VEGFR2^+$ circulating endothelial progenitors and $CD34^+CD133^-$ mature endothelial cells shed from the vessel wall, but their frequency is extremely low ($<0.01\%$ of PBMCs) [4, 33, 34]. Moreover, the depletion of $CD34^+$ cells from PBMCs before the generation of MOMCs did not affect the yield of ELCs. Although we could not entirely exclude the possibility that cell fusion was partly responsible for the phenotypic change of human MOMCs in the in vivo tumor vascularization model, we believe that the involvement of cell fusion in our observations is unlikely, because endothelial cells expressing both mouse and human CD31 were hardly ever detected in the tumor blood vessels.

MOMCs are derived from circulating $CD14^+CD34^-$ monocytes [10], but their detailed origin is unknown. Recently, two populations of circulating cells with the capacity to differentiate into endothelial cells were reported by two investigator groups [27, 35]. MOMCs appear to correspond to early EPCs, which show $CD14^+$ spindle-shape morphology and rapid differentiation into endothelial cells. However, MOMCs have limited proliferative capacity: this characteristic might be acquired through differentiation into MOMCs without angiogenic stimulation. On the other hand, Romagnani et al. have reported that circulating $CD14^+CD34^{low}$ cells, which are not detected by a standard flow cytometry or magnetic bead-based sorting but can be detected by the highly sensitive antibody-conjugated magnetofluorescent liposomes technique, exhibit both phenotypic and functional features of pluripotent stem cells [36], suggesting that $CD14^+CD34^{low}$ cells are the origin of MOMCs.

Emerging evidence suggests that the transplantation of various distinct cell types containing potential endothelial progenitors, obtained either by isolation or ex vivo cultivation from the bone marrow or peripheral blood, augments the neovascularization of ischemic tissue [25, 37]. In initial pilot studies, the introduction of autologous cells derived from the bone marrow

or peripheral blood induced a therapeutic improvement in the blood supply to ischemic tissue [38, 39]. Presently, a variety of cell types, including unfractionated bone marrow cells, bone marrow-derived $CD133^+$ cells, circulating $CD133^+$ cells mobilized by granulocyte colony-stimulating factor, and ELCs generated in the EPC culture, have been proposed as transplantable cells for therapeutic neovasculogenesis, but it remains unclear which cell source is the best for therapeutic cell transplantation to promote organ vascularization in terms of efficacy and safety. Cell therapy using MOMCs has some advantages over the currently proposed strategies using other cell sources, since peripheral blood, without progenitor cell mobilization treatment, is a relatively obtainable and safe source of autologous cells. Theoretically, $>10^8$ MOMCs could be prepared by leukapheresis [10], although the number of MOMCs requiring effective vascular regeneration therapy is unknown. Further studies comparing the clinical potential of various endothelial progenitors to restore long-lasting organ vascularization and function are necessary.

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Disclosures

The authors indicate no potential conflicts of interest.

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